UNIVERSIDADE FEDERAL DE UBERLÂNDIA

FACULDADE DE MEDICINA VETERINÁRIA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

EDSON CAMPOS VALADARES JUNIOR

REPLICAÇÃO E EFEITOS DO FAGO M13 DA BIBLIOTECA DO *PHAGE DISPLAY* EM FRANGOS DE CORTE

Uberlândia

2022

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Replicação e efeitos do fago M13 da biblioteca do *phage display* em frangos de corte

Dissertação apresentada ao Programa de Pós-graduação em Ciências Veterinárias da Faculdade de Medicina Veterinária da Universidade Federal de Uberlândia como requisito parcial para obtenção do título de mestre em Ciências Veterinárias.

Área de concentração: Saúde animal

Orientador: Profª Drª Belchiolina Beatriz Fonseca

Coorientador: Profª Drª Aline Santana da Hora

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Área de concentração: Saúde Animal

Uberlândia, 30 de agosto de 2021

Banca Examinadora:

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Belchiolina Beatriz Fonseca – Doutora (FAMEV-UFU)

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ATA DE DEFESA - PÓS-GRADUAÇÃO

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Reuniu-se por Videoconferência (meio eletrônico), da Universidade Federal de Uberlândia, a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em Ciências Veterinárias, assim composta: Professores Doutores: **Roberta Torres de Melo - UFU; Álvaro Ferreira Júnior - UFG; Belchiolina Beatriz Fonseca** orientador(a) do(a) candidato(a).

Iniciando os trabalhos o(a) presidente da mesa, Dr(a). Belchiolina Beatriz Fonseca, apresentou a Comissão Examinadora e o candidato(a), agradeceu a presença do público, e concedeu ao Discente a palavra para a exposição do seu trabalho. A duração da apresentação do Discente e o tempo de arguição e resposta foram conforme as normas do Programa.

A seguir o senhor(a) presidente concedeu a palavra, pela ordem sucessivamente, aos(às) examinadores(as), que passaram a arguir o(a) candidato(a). Ultimada a arguição, que se desenvolveu dentro dos termos regimentais, a Banca, em sessão secreta, atribuiu o resultado final, considerando o(a) candidato(a):

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O competente diploma será expedido após cumprimento dos demais requisitos, conforme as normas do Programa, a legislação pertinente e a regulamentação interna da UFU.

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“Esquecer é uma necessidade. A vida é uma lousa, em que o destino, para escrever um novo caso, precisa de apagar o caso escrito.”

(Machado de Assis)

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RESUMO

O *phage display* (PD) é uma tecnologia para seleção de peptídeos miméticos e ligantes com objetivos terapêuticos, vacinais e diagnósticos. Após a seleção via PD, os peptídeos são sequenciados e caracterizados para serem usados de acordo com o objetivo. Nesse trabalho se propõe uma nova abordagem em que o fago M13 apresentando os peptídeos selecionados poderão ser utilizados diretamente como ligante ou mimético em aves. Frangos de corte foram inoculados com o fago M13 e a *Escherichia coli* E R2738 (ECR) infectada com o fago M13 via oral nas idades de 2, 8 e 15 dias de vida. Nas idades de 7, 14, 21, 28 dias de vida as fezes das aves foram coletadas e o fago M13 foi quantificado. Os fagos foram encontrados nas fezes em todas as idades avaliadas no grupo inoculado apenas com ECR infectada com o fago M13. A presença do fago M13 ou da ECR não resultou em alterações na saúde das aves, alterações macroscópicas no intestino ou outros órgãos ou no ganho de peso das aves. O ensaio de ELISA mostrou que os níveis de IgY foi similar em todos os tratamentos o que indica que o vírus pode ser usado em aves até 42 dias sem uma resposta imune humoral contra o fago. Esse trabalho mostra que quando a ave é inoculada com ECR infectada com o fago M13, o vírus pode replicar no trato intestinal da ave sem alterar a saúde do animal ou estimular a resposta imune humoral. Essa abordagem permite o uso dos fagos da biblioteca do PD expressando peptídeos selecionados para controle de patógenos no trato intestinal das aves.

Palavras-chave: peptídeos, controle, ligantes, miméticos.

ABSTRACT

Replication and effects of M13 phage from the phage display library in broiler chickens

The phage display (PD) is a technology for selecting mimetic peptides and ligands for therapeutic, vaccine, and diagnostic purposes. After selection via PD, the peptides are sequenced and characterized for use. This work proposes a new approach in which the M13 phage displaying the selected peptides can be used directly as a binder or mimetic in birds. Broiler chickens were inoculated with phage M13 and Escherichia coli E R2738 (ECR) infected with phage M13 orally at 2, 8 and 15 days of age. Bird feces were collected at the ages of 7, 14, 21, and 28 days of life, and the M13 phage was quantified. Phages were found in feces at all ages evaluated in the group inoculated only with ECR infected with M13 phage. The presence of M13 phage or ECR did not result in changes in bird health, macroscopic changes in the intestine or other organs, or in bird weight gain. The ELISA assay showed that IgY levels were similar in all treatments indicating that the virus can be used in birds for up to 42 days without a humoral immune response against the phage. This work shows that when the bird is inoculated with ECR infected with the M13 phage, the virus can replicate in the bird's intestinal tract without altering the animal's health or stimulating the humoral immune response. This approach allows the use of PD library phages expressing selected peptides to control pathogens in the intestinal tract of birds.

Keywords: peptides, control, ligands, mimetics.

Sumário

INTRODUÇÃO

A avicultura é um dos setores mais lucrativos para o Brasil e, portanto, atender aos critérios internacionais de qualidade microbiológica e garantir a segurança do consumidor é crucial para que o país se mantenha em sua posição de destaque no mercado (Nääs et al. 2015). Inserido nos riscos microbiológicos, a multirresistência bacteriana aos antimicrobianos representa um grande risco para a saúde pública e sérios prejuízos econômicos, já que bactérias resistentes nos animais podem ser transferidas aos humanos (Ben et al. 2019). Com isso, novas alternativas terapêuticas preventivas para o controle de microrganismos vêm sendo usadas. No entanto, ainda há necessidade de expansão e a diversidade de tecnologia que substituam os antimicrobianos (Baker et al. 2018).

A técnica do *phage display* (PF) têm sido empregada na seleção de peptídeos utilizados em estudos de proteção contra várias doenças pela capacidade de interações moleculares de uma única molécula ou célula, sem conhecimento prévio sobre a natureza da mesma (Huang et al. 2012). Nessa tecnologia, uma das configurações é quando uma vasta bibliotecas de peptídeos recombinantes, fusionados a proteína PIII do fago se ligam a antígenos e anticorpo (Smith 1985). As ligações permitem então, a seleção de peptídeos que poderiam ser usados para diferentes estratégias, incluindo controle e prevenção de patógenos (Henry et al. 2015a).

Após a seleção dos clones dos fagos selecionados, usualmente os peptídeos são sequenciados e assim sintetizados para serem usados como prevenção ou terapia (Kuzmicheva and Belyavskaya 2016). Mas uma outra abordagem poderia ser explorada que é o uso dos fagos selecionados (Costa et al. 2014). A vantagem do uso do fago expressando os peptídeos é o tamanho da molécula que permitiria melhor interação e/ou maior estímulo a resposta imune visto que o peptídeo é uma molécula pequena.

Evidências indicam que epítopos expressos em bacteriófagos selecionados por PD (em outros bacteriófagos que não o M13) podem estimular eficientemente uma resposta imune celular (Castel et al. 2011). Nesse contexto a administração de bacteriófagos M13 selecionados expressando peptídeos imunogênicos específicos de agentes patogênicos poderia ser uma alternativa para controle de microrganismos intestinais na avicultura.

A fagoterapia utilizando bacteriófagos selecionados que expressem peptídeos miméticos que auxiliam no controle de doenças ainda é um tema não explorado na avicultura mundial. Até o momento não se sabe sobre a inocuidade do fago M13 (comumente utilizado na tecnologia do PD) nem tampouco sobre sua replicação e interação com microbiota. Trabalhos nessa linha poderiam aumentar o leque do uso dos bacteriófagos para diferentes objetivos dentro da medicina veterinária. Assim, o objetivo desse trabalho é avaliar se o fago M13 pode permanecer viável no trato intestinal das aves e se causa danos às aves.

CAPÍTULO 1

REVISÃO DE LITERATURA

1- *Phage display*

O PD é uma técnica baseada na ligação de peptídeos a um alvo de interesse com alta afinidade e especificidade. George Smith (1985), utilizou bacteriófagos modificados geneticamente para expressão de peptídeos na superfície do capsídeo dos vírus, com isso foi possível a aplicação em diferentes alvos, para formar pares receptores-ligantes de interesse. A codificação do peptídeo está presente no genoma do bacteriófago, sendo possível a identificação e recuperação de uma partícula viral única.

A biblioteca de bacteriófagos consiste na inserção de genes responsáveis pela codificação de milhões de peptídeos que são expressos na superfície viral, fusionados em proteínas estruturais do capsídeo. Quanto mais diversificados os peptídeos da biblioteca, maior são as probabilidades de encontrar alvos que possuem alta afinidade com o alvo de interesse (Smith 1985).

A técnica de PD consiste em bibliotecas de peptídeos randômicos que possuem uma complexidade de 10^9 clones independentes, que são utilizados para selecionar sequencias peptídicas através de *biopanning*. Esta grande variação (10^9 ou mais) é suficiente para codificar grande parte, se não todas as possíveis combinações de sequências peptídicas. Estes peptídeos, são capazes de mimetizar estruturas conformacionais e epítopos descontínuos ou contínuos (Barbas, C. F. I.; Burton, D. R.; Scott, J. K.; Silverman 2001). É sem dúvida uma técnica promissora na criação de novas vacinas (Aghebati-Maleki et al. 2016).

Com auxílio de uma técnica denominada *biopanning*, é possível a apresentação da biblioteca de bacteriófagos a moléculas-alvo de interesse que são imobilizadas em algum suporte como placas de imunoensaio. Com um conjunto de lavagens sucessivas, é possível a seleção de clones que possuem maior afinidade de ligação com os peptídeos expressos pelo vírus e o alvo. Esses clones com alta capacidade de ligação, podem ser recuperados por um processo de eluição, composto por tampões ácidos com elevada força iônica que rompem a ligação peptídica formada ((Barbas, C. F. I.; Burton, D. R.; Scott, J. K.; Silverman 2001). Posteriormente, o ciclo de seleção, eluição e amplificação de três a cinco vezes. Com a ajuda de titulações e realização de ELISA, é possível monitorar a quantidade de fagos que entraram no ciclo e foram obtidos após o processo de seleção, e analisar individualmente os clones quanto a capacidade de ligação com o alvo. Os peptídeos dos clones obtidos após o final do processo, podem ser posteriormente sequenciados e usados de forma isolada para avaliar afinidade e especificidade em diversas aplicações (Barbas, C. F. I.; Burton, D. R.; Scott, J. K.; Silverman 2001).

O bacteríofago mais utilizado é o M13 que é um vírus filamentoso, com genoma circular DNA- fita simples com cerca de 6400 bases, composto por proteínas pIII, pVI, pVII, pVIII e pIX. As proteínas mais utilizadas para expressão de peptídeos são a pIII e pVIII, apesar de ser possível a apresentação de moléculas em todas as cinco. Pertencem à família *Inoviridae*, gênero *Inovirus*, e infectam enterobactérias como a *Escherichia coli*, podendo ser infectada sem danos estruturais devido ao ciclo lisogênico desse vírus (Russel 1991).

A proteína pIII é responsável pela ligação do fago ao pilus sexual da bactéria, sendo fundamental para a reprodução viral. É composta por 3 a 5 cópias, e suporta proteínas fusionadas de até 450 aminoácidos, como frações de anticorpos ScFv. Já a proteína pVIII, não possui a capacidade de fusão de grandes sequências de aminoácidos, apresenta cerca de 2800 cópias e reveste o capsídeo viral, dando forma cilíndrica ao vírus (Russel 1991).

O fago M13 possui o gene LacZ, sendo possível fazer a diferenciação de colônias bacterianas infectadas (azul) e colônias não infectadas (brancas) ou infectadas com fago selvagem. A bactéria é capaz de produzir a enzima β-galactosidase, que com auxílio do IPTG, é capaz de degradar o X-Gal, responsável pela coloração azul da célula bacteriana (Barbas (Barbas, C. F. I.; Burton, D. R.; Scott, J. K.; Silverman 2001).

Os peptídeos podem ser expressos tanto na forma linear, como conformacionais. Porém, as imunoglobulinas possuem preferência de interação por epítopos não lineares, com isso, é recomendável o uso de bibliotecas que possuem cisteínas introduzidas nas extremidades da sequência randômica para conferir uma estrutura circular do peptídeo pela formação de uma ponte dissulfeto (Smith and Petrenko 1997).

As bibliotecas de peptídeos comerciais são constituídas por monômeros (-mer), geralmente possuem de sete ou 12 resíduos de aminoácidos, que produzem grande variedade de sequências únicas. Existem vários tipos de bibliotecas que podem ser utilizadas atualmente, em diversas aplicações. Como bibliotecas de anticorpos, receptores de células T (TCR) e de peptídeos-MHC (de Almeida et al. 2015).

2. O intestino da ave e a fagoterapia

Fagos selvagens que infectam bactérias intestinais tem um papel importante na microbiota e podem reagir com o sistema imune criando um balanço entre saúde e doença (Gutiérrez and Domingo-Calap 2020). A fagoterapia tradicional é uma alternativa na redução do uso de antimicrobianos (Lin et al. 2017). A inclusão de coquetel de bacteriófagos que infectam diferentes bactérias aumenta o peso do frango durante os primeiros dias além de promover a proliferação de microrganismos benéficos no trato intestinal (Upadhaya et al. 2021). No entanto, algumas desvantagens podem representar um problema no uso de fagos como o desenvolvimento de uma resposta imune contra esses vírus (Hodyra-Stefaniak et al. 2015), a transferência de genes de virulência e resistência que fagos líticos podem levar para diferentes bactérias (Wernicki et al. 2017).

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CAPÍTULO 2

Journal: APPLIED MICROBIOLOGY AND BIOTECHNOLOGY

Alternative use of *phage display*: Phage M13 can remain viable in the intestines of poultry without causing damage

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Abstract

Phage display (PD) is a tool for developing new molecules to control pathogens. Peptides selected by PD are commonly synthesised and tested, but the use of phage M13 displaying the selected peptides as a direct biding in the intestinal tract has not yet been tested. This study evaluated whether phage M13 can remain viable in the chicken gastrointestinal tract and whether it causes injury or humoral immune response. We inoculated phage M13 or *E. coli* ER2738 (ECR) infected with M13 into birds at different ages. We found the virus in faeces at 5 or 13 days after inoculation, just when it infected the ECR. The presence of phage M13 or ECR did not result in gut injuries and had no impacts on weight gain and bird health. Furthermore, the levels of IgY were similar in all treatments, which indicates that the virus can be used in chicken until 42 days without being recognised by the immune system. This work provides a scientific basis for the use of PD as a tool in numerous applications to control different pathogens.

Keywords: control, ELISA, peptides, Chickens

Key Points

- Phage M13 remains viable in the bird's intestine if inoculated with *E. coli* ER2738
- Bacteriophage M13 does not damage the chicken gut
- Phage M13 remains in the gut without leading to a humoral response up to 42 days.

Introduction

The search for antibiotics replacement is still an important topic. The increase in the use of antibiotics in poultry production is correlated to an increased antibiotic resistance of bacteria (Muaz et al. 2018), leading to complications in the treatment of bacterial diseases in humans and animals. In this sense, several alternatives to antibiotics are used in poultry production with varying success, such as probiotics, prebiotics, symbiotics, essential oils, phytotherapy, organic acids, enzymes, bacteriophages and peptides (Gadde et al. 2017). In addition, the use and study of new vaccines are critical tools to reduce the among of antibiotics in poultry production.

The phage display (PD) technique can contribute to the replacement of antibiotics; it is used to select, characterise and identify peptides (Saw and Song 2019). The bacteriophage M13 is a filamentous phage that displays peptides linked to the minor coat protein III gene. Based on the vast library displayed in M13, peptides can be selected and purified; a high amount of the bacteriophage has been replicated in a modified *E. coli* (Barbas et al. 2001). This provides high-affinity peptides, which can be employed in the development of peptides to vaccines (Ma et al. 2019) and in therapeutics strategies (Mimmi et al. 2019).

After the selection by PD, the peptides displayed by phage M13, are sequenced, characterised and synthesised for later use. In the poultry industry, oral vaccination or treatment can be adequate because of the group approach. Additionally, some microorganisms, such as paratyphoid *Salmonella, Campylobacter* and *Eimeria*, are usually limited to the gastrointestinal tract in birds, which requires prevention or oral therapy. In the context of vaccines, in some cases, peptides cannot stimulate the immune response because of the limitations concerning immunogenicity (Malonis et al. 2020). Therefore, although treatment with antimicrobial peptides can be efficient (Wang et al. 2016), alternative methods should be developed.

According to a previous study, the epitopes expressed in bacteriophages selected by PD can stimulate the immune response (Díaz-Valdés et al. 2011). However, the use of the bacteriophage M13 displaying selected peptides has not yet been investigated in the poultry gut, and therefore, we do not know whether bacteriophage M13 can survive in the gastrointestinal tract and be innocuous to poultry. A deeper knowledge of this issue can facilitate other studies on phage M13 displaying the peptides. Being a larger structure than the purified peptide, phage M13 displaying selected peptides may stimulate the local immune response and/or bind to microorganisms more successfully. In this context, we evaluated the viability and innocuity of the wild M13 bacteriophage in the intestine of chickens, with the aim to obtain an alternative for future studies in which these phages are used in therapies or vaccines.

Methods

A total of 140 chicks (Hy-Line w-36®) from an industrial incubator were grown in the Ornitopathology Laboratory of the Federal University of Uberlândia, Brazil. The animals were housed on the same day of birth and kept until 42 days of age on the floor in previously autoclaved wood shavings. Water and food were provided *at libitum* during the entire rearing period; temperature and humidity were controlled according to the age. The final density was 7 birds/m^2. The nutritional level of the feed is shown in Supplemental Table 1.

Management, sampling and euthanasia were carried out following the guidelines of the Committee on Ethics in the Use of Animals of the Federal University of Uberlândia (nº 118/18).

Inoculum preparation and titration

The inoculum was prepared after the incubation of one colony of *Escherichia coli* ER2738 (ECR) at 37ºC in *Luria Bertani* - (LB - Tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L) (Kasvi) with tetracycline (Sigma Chemical Co., 20 mg/mL) under agitation until the *earlylog* (OD600 ~0.3). Part of the inoculum was stored, and the other part was inoculated with the 10 log PFU of phage M13 incubated at 37°C for 4 hours under agitation. The culture was centrifuged at 15,000 x g for 10 minutes, and the pellet containing ECR infected by the M13 phage was used as inoculum. The supernatant was transferred to a tube containing PEG/NaCl (20% polyethylene glycol 8000, Fluka, and 2.5 M NaCl Neon – sterile solution), incubated at 4°C for 16 h and centrifuged. Finally, the phage pellet was resuspended with sterile PBS and used as phage inoculum.

Infected and non-infected bacteria were quantified on an LB agar plate after several dilutions. For titrating of the phage M13, the ECR was previously prepared in LB and incubated at 37ºC under agitation up to *mid-log* (OD600 ~0.5). A total of 200 uL of LB containing *E. coli* ER2738 (ECR) was added to each dilution of the phage for 5 minutes, and subsequently, each dilution was added to TOP agarose (LB 20 g/L, MgCl 2 $6H_2O$ 1 g/L, agarose 7 g/L) and labelled on LB agar plates.

Inoculation in animals and collection of biological samples

The birds were inoculated at 2, 8, 15 days of age, receiving the treatment intraoesophagally. They were divided into five groups (28 birds/group), receiving the following treatments: Group G1 (G1) received 10 log PFU/bird of bacteriophage M13 in PEG and PBS;

Group G2 (G2) was inoculated with 5 to 8 log CFU/bird of ECR infected with bacteriophage M13 diluted in PBS; Group G3 (G3) was inoculated with 6 to 8 log CFU/bird of ECR diluted in PBS; Group G4 (G4) received PEG (bacteriophage control); Group 5 (G5) was inoculated with PBS (negative control). The exact amounts of each inoculum of ECR are listed in Supplementary Table 2.

At 7, 14, 21 and 28 days of age, three birds of each group were euthanised, and we evaluated intestine integrity visually and collected faeces directly from the rectum to count the phages. We performed histomorphometry at 35 and 42 days of age, after macroscopic analyses. At 42 days of age, the blood of three birds was collected directly from the brachial vein (2 mL of blood), the gut was macroscopically analysed. The remaining birds were used only for zootechnical analysis.

Phage count in faeces

In the Nanobiotechnology Laboratory at the Federal University of Uberlândia, the faeces were weighed and diluted in 5,000 uL PBS (dilution 1:10), followed by homogenisation. Diluted faeces were filtered, and the remaining liquid was centrifuged at 15,000 x g for 10 minutes. The supernatant was spiked with 1/6 of PEG and stored in a cold chamber overnight (+/-4^{0}C). Subsequently, the sample was centrifuged at 15,000 x g for 10 min, the pellet was resuspended, and 30 uL was placed in 9,970 uL of LB; six serial dilutions were performed in LB. For labelling, the ECR was previously prepared in LB incubated at 37ºC under agitation up to *mid-log* (OD600 ~0.5). A total of 200 uL of LB containing ECR was added to each dilution of the phage for 5 minutes. Each dilution was added to TOP agarose and labelled in LB agar added to IPTG (isopropyl β-D-1-thiogalactopyranoside - Ludwig Biotec) (0.5 mM) in *mid-log* (OD600 ~0.5) and X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside - Ludwig Biotec) (40 µg/mL), followed by incubation at 37ºC for 24 hours. The blue colonies (where 15–50 colonies grew) on the plates were counted.

DNA extraction

The blue colonies isolated from the faeces were amplificated in ECR and centrifugated at 15,000 x g RPM for 10 minutes. The supernatant was added to 1/6 of PEG overnight, and subsequently, the sample was centrifugated at 15,000 x g for 10 minutes; the obtained supernatant was resuspended in iodide buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA and 4 M NaI). The tubes were agitated for 40 seconds at ambient temperature, and 250 uL of ethanol was added. After 10 minutes, the phages were centrifuged at 3,700 x g for 10 minutes at 20˚C, and the supernatant was removed. The DNA was washed with ethanol and centrifugated as described above. The precipitated DNA was diluted in nuclease-free water.

PCR for M13 confirmation

Polymerase chain reaction (PCR) was performed to confirm that the blue colonies isolated from faeces were M13. The reactions were conducted for a final volume of 10 µL, containing 1 µL of the DNA sample, 10 pmol/µL of each primer (forward: 5´-GTAAAACGACGGCCAG-3', reverse: 5´-CAGGAAACAGCTATGAC- 3') (Invitrogen), 0.25 µl of 20 mM of the mix of dNTPs (Invitrogen), 0.25 µL of Taq (5U/µL) (Invitrogen). The samples were submitted to the following amplification cycles: initial denaturation at 95°C for 5 minutes, 35 cycles at 94°C for 40 seconds, 56°C for 40 seconds, 72°C for 50 seconds, and a final extension at 72°C for 10 minutes. All PCR reactions were performed on a thermal cycler (Eppendorf), and the amplified products were separated via 1.5% agarose gel electrophoresis. The gel was stained with Syber Safe (Invitrogen) and visualised in UV translucent vessels.

Sequencing of M13 phage

To increase the certainty that the phages isolated were M13, we sequenced four blue colonies, using 200 ug of DNA, 5 pmol of primer -96 gIII (5'-OH CCC TCA TAG TTA GCG TAA CG-3' - Biolabs) and Premix (DYEnamic ET Dye Terminator Cycle Kit – Amersham Biosciences). The 35 cycles were performed in a plate thermocycler (MasterCycler-Eppendorf) under the following conditions: denaturation (95°C, 20 seconds), annealing (50°C, 40 seconds) and extension (60°C, 60 seconds). The amplified DNA was resuspended in 10 µL of dilution buffer (DYEnamic ETDye Terminator Cycle Kit – Amersham Biosciences), and the sequences were read on a MegaBACE 1000 automatic sequencer (Amersham Biosciences). The DNA sequences were analysed using the equipment's software (Sequence Analyzer, Base Caller, Cimarron 3.12. Phred 15). Immediately after this pre-analysis, the vector sequences were removed, and only those inserted with perfect residues were translated.

After sequencing, the DNA sequences were translated using the DNA2PRO12 program. This program automatically locates the position of the insert, translates it, and indicates any possible error in the sequence, such as unexpected codons or near-sequence errors (http://relic.bio.anl.gov/dna2pro12.aspx).

Performance and macroscopic and gut macroscopic change analysis

The birds were monitored two times a day, and the typical behaviour (activity, scratching, opening the wings when bathing in the shavings) as well as the faeces characteristics were evaluated. The birds were weighed at 1, 7, 35 and 42 days of age. Uniformity was calculated, and feed intake and conversion were calculated based on the weighed rations. At 7, 14, 21, 28 and 42 days of age, three birds of each group were euthanised and necropsy was performed, observing all organs in the cloacal pouch, thymus, spleen, liver, oesophagus, crop, gizzard, intestine and cecal tonsils.

Intestine histomorphometric analyses

At 35 and 42 days of age, the ileum and cecum of the G2 and G5 were collected for histomorphometric analysis. The fragments were fixed in 10% buffered formalin and processed to prepare histological slides stained with haematoxylin and eosin (HE) (Tolosa et al. 2003). Ileum villi height and ileum and cecum crypt depth were measured using the ImageJ morphometry program.

ELISA

To assess whether the phages were able to produce a systemic immune response, ELISA was performed at 42-day-old birds as described elsewhere (Santos et al. 2018), with modifications. The microtitration plates were sensibilised with 1 μg/well of phage M13 diluted in 50 mM bicarbonate buffer (pH 8.6) for 16 hours at 4°C. After three washes with PBS-T (PBS + Tween 20 at 0.05%), the plates were blocked with PBS-T with 5% skim powdered milk for 1 hour at 37ºC and subsequently washed three times with PBS-T, followed by incubation with different bird serum at different dilutions (1:50 and 1:100) at 37ºC for 1 hour. The controls were performed without phages or serum. After five washes with PBS-T, the anti-IgY at 1:5000 was added, followed by incubation at 37ºC for 1 hour. The wells were washed five times, and the ligation antigen/antibody was detected by the addition of ortho-phenylenediamine (OPD) at 1 mg/mL with 3% H_2O_2 (Sigma Chemical Co.). The reaction was stopped by the addition of 4N sulfuric acid. Reactivity was determined in a plate reader (Titertek Multiskan Plus, Flow Laboratories, USA) at a wavelength of 492nm. The results represented the absorbance in wells sensitised with phage subtracted from that of the control wells (not sensitised with phages).

Statistical analysis

We performed analysis of variance (ANOVA) followed by Tukey's test to analyse the ELISA and phage counts or test t to histomorphometric, with a 95% confidence interval, using the GraphPad Prism 9.2. We used the GLM (General Linear Models) procedure of the SAS statistical package (2008) to weigh gain analysis.

Results

It is necessary the ECR to M13 viability in poultry

Quantification of plaque-forming units (PFUs)

We inoculated phage M13 and ECR infected with phage M3 into 2-, 8- and 15-day-old birds. However, we only recovered M13 in the faeces of the group treated with ECR infected by phage M13 (Group 2) (Table 1).

PCR of t phage M13 suspect colonies

As we had no information about the microbiota of the poultry studied, we performed a PCR to confirm that the colonies found were the M13 phage. Based on the results, the phages found amplified with the phage primers used.

Sequencing the M13 phage

Sequencing was performed to confirm that the colonies were the M13 phages from the PD library. The sequences of all four blue colonies were identical, and the analysis confirmed the M13 phage from the PD library. In all results, the sequence 'CTATTCTCAC', located in the P3 insert region of the phage M13, was present.

The presence of phage M13 in the gut does not change zootechnical parameters or cause damage to poultry

The birds were monitored twice daily. We observed no changes in their behaviour and health. Via necropsy of euthanised animals, we found no organ changes. During the experiment, the mortality rate was zero; there were no changes in weight and average daily feed intake (Table 2).

The presence of M13-infected ECR did not change the intestinal histomorphometry of ileum and cecum

We performed histomorphometric analyses of the ileum and cecum. No microscopic changes related to inflammation, degeneration or necrosis were observed. Furthermore, ileum villi heigh or the depth of the cecum and ileum crypts did not change (Fig. 1).

Birds did not develop a humoral immune response against M13 phage

To assess whether the phages could induce a systemic immune response, ELISA was carried out in 42-day-old birds. There were no differences in the ELISA results among the groups (Figure 2).

Discussion

Alternatives to the use of antibiotics in animal production are urgently needed. In this sense, several molecules are tested and used in poultry production. The PD technology allows the selection of peptides with high affinity and specificity to the antigen of interest. It is also suitable for applications in different research areas, being of paramount importance for innovation in diagnosing and treating various diseases (Henry et al. 2015).

Based on our results, phage M13 is viable inside the poultry gut and can replicate, but only when inoculated with ECR. Our hypothesis about the replication is based on the quantification of M13 per gram of faeces. Five or 6 days after inoculation, the amounts of the phage M13 per gram of faeces were 0.91, 1.25, 1.14 log PFU above the inoculated amount of ECR infected by M13 (Supplementary Table 2). Besides, 13 days after the last inoculation (at 28 days of age), the birds had 1.2 log PFU/g of faeces above the inoculated amount of ECR infected by M13 (Supplementary Table 2). Under laboratory conditions, M13 takes 5 minutes to invade the bacterium (Barbas et al. 2001). As we inoculated the infected bacteria, the phages then replicated within the bacteria and reached the intestinal tract, probably performing other cycles. We collected samples for analysis at 42 days, but unfortunately, an equipment issue did not allow us to obtain the results.

Interestingly, we found no M13 in birds inoculated without bacterium. Phage M13 needs an *E. coli* with sexual pili to replicate (Chung et al. 2011). Although *E. coli* commonly occurs in the microbiota on the intestine, not all *E. coli* have sexual pili (Iuchi et al. 1989). The specificity of the phage M13 to ECR is a critical point since this feature does not allow this phage to spread the resistance gene from the sexual pili.

Bacteriophage M13 can remain viable in the gut poultry; we recovered free phages from the faeces when inoculating ECR infected with M13. However, we do not know whether the M13 phage was found in the faeces (when inoculated alone) because it did not survive at the conditions of the gastric tract or because there was no *E. coli* with sexual pili in the intestine of the poultry. However, in practical terms, if we are going to use phage M13 displaying peptides binding to some antibody or antigen in the intestine, it is necessary to use *E. coli* expressing sexual pilli. Or else, technologies should be applied to ensure mucoadhesion and/or delivery and persistence of the M13 phage in the gastrointestinal tract over a long period.

When we started the experiment, our main concern was to isolate lactose-fermenting bacteria from the microbiota or even other phages that could infect *E. coli*. To ensure this, we performed several rounds of phage purification, PCR of 100% of the countable colonies and sequenced four of them. The PCR results showed that we had isolated phage M13. We used a primer that loops in the PIII-fused amino acid insert region in the sequencing, which made us more confident that we could isolate the inoculated phages from the faeces.

When analysing a probiotic microorganism or even a phage in animals, it is essential to assess virulence. Therefore, we evaluated several bird parameters such as behaviour, weight gain, morbidity, mortality, as well as macroscopic and microscopic lesions. Our analysis showed that the phage is safe for the birds because neither zootechnical, macroscopic, microscopic and histomorphometric changes were observed (Table 2, Fig. 1). One of the important issues with phage therapy is the development of immunity against the phage (Krut and Bekeredjian-Ding 2018); therefore, we performed the ELISA at 42 days of age. Based on the results, there was no increase in antibodies against M13 in the treated group, indicating that in birds with a short cycle, phage M13 can be used without the bird's organism producing antibodies that inactivate this virus.

Inoculation of ECR infected with bacteriophage M13 allows this phage to survive in the intestines of chickens without harming them. This could be a new alternative to the use of PD for the control of pathogens in poultry.

Statements and Declarations

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Data Availability Statement: Data are contained within the article or the Supplementary Material. Complete data will be made available upon request.

Conflicts of Interest: The authors declare that they have no conflicts of interest.

Consent for publication: All authors have read and agreed to the final version of the manuscript.

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Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Management, sampling and euthanasia were carried out following the guidelines of the Committee on Ethics in the Use of Animals of the Federal University of Uberlândia (nº 118/18).

Authors' Contributions

LRG and BBF planned the research. FAAS, LRG, BBF, ECVJ, LMB designed the experiment. BBF, PLFN, ELP, LVCG, ECVJ collected the samples. LVCG nad BBF planned the conditions. FAAS, TBF, ECVJ, PLFN, BBF, EPM performed the microbiologic analysis. FAAS, BBF, PLFN, ASH, ECVJ performed the PCR and carried out the sequencing and bioinformatics. LVCG, PLFN, ECVJ determined the performance parameters. BBF, ECVJ, LVCG carried out the behavior analysis. BBF and ECVJ made the necropsy. FAAS and PLFN performed the ELISA. BBF and LRGF evaluated the data. BBF, ECVJ, ASH and FAAS wrote the manuscript.

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Figure captions

Figure 1. Ileum villi height and ileum and cecum crypt depth (um) in 42-day-old birds inoculated with M13-infected ECR.

G2: Group inoculated with *E. coli* ER2738 infected by phage M13; G5: Group inoculated with PBS. DIC: depth of the ileum crypts, IVH: ileum villi height, CCD: cecum crypt depth. T tests were performed between G2 and G5 to DIC or IVH or CCD ($p \leq 0.05$). There was no difference in the DIC, IVH or CCD between G2 and G5.

Figure 2. Levels of antibody (measured by absorbance) anti-M13 in birds of the different treatment groups.

G1: Group inoculated with phage M13; G2: Group inoculated with *E. coli* ER2738 infected with phage M13; G3: Group infected by *E. coli* ER2738; G4: Group inoculated with PEG; G5: Group inoculated with PBS. Control: plate sensitised with phage without serum. No differences were found ($P > 0.05$) by Tukey´s test.

Tables

Table 1. Mean values of M13 phages per gram of bird faeces (log PFU/g) in different groups and at different ages.

Age/Groups	G1	G2	G3	G4	G5
7	0.00 (+/-0.00)[a]	4.84 (+/-0.82) [b]	0.00 (+/-0.00) [a]	0.00 (+/-0.00) [a]	0.00 (+/-0.00) [a]
14	0.00 (+/-0.00) [a]	5.27 (+/-0.25) [b]	0.00 (+/-0.00) [a]	0.00 (+/-0.00) [a]	0.00 (+/-0.00) [a]
21	0.00 (+/-0.00) [a]	5.77 (+/-0.57) [b]	0.00 (+/-0.00) [a]	0.00 (+/-0.00) [a]	0.00 (+/-0.00) [a]
28	0.00 (+/-0.00) [a]	4.6 (+/-0.60) [b]	0.00 (+/-0.00) [a]	0.00 (+/-0.00) [a]	0.00 (+/-0.00) [a]

Values represent mean and standard deviation. G1: group inoculated with phage M13. G2: Group inoculated with *E. coli* ER2738 infected by the phage M13; G3: Group infected with *E. coli* ER2738; G4: Group inoculated with PEG; G5: Group inoculated with PBS. The birds of G1 were inoculated at all ages (10 PFUs/bird). The birds of G3 were inoculated at 2 days (5.04 log

CFU/animal), 8 days (6.64 log CFU/animal) and 15 days of age (6.69 log CFU/animal). Different letters represent statistical difference (P > 0.05) by grouped ANOVA.

Table 2. Mean daily weight gain of birds (MDWG), mean daily feed consumption (MDFC) and feed conversion (FA) of bords of the line Hy-line W-36® at 1–7 days, 8–35 days and 36–42 days of age.

Tratament[1]	**1–7 days old (g/day)**			**8–35 days old (g/day)**			**36–42 days old (g/day)**		
	MDWG	**MDFC[2]**	**FA[2]**	**MDWG**	**MDFC[2]**	**FA[2]**	**MDWG**	**MDFC[2]**	**FA[2]**
G 1	14.29	19.80	1.39	31.76	46.20	1.45	57.37	139.46	2.43
G 2	15.59	19.83	1.27	30.64	46.16	1.51	59.78	132.89	2.22
G 3	15.61	19.58	1.25	30.00	50.74	1.69	47.87	132.43	2.77
G 4	14.97	20.42	1.36	27.88	41.10	1.47	68.09	117.17	1.72
G 5	15.86	18.89	1.19	31.16	45.04	1.45	61.99	135.17	2.18
Mean	15.26	19.70	1.29	30.29	45.85	1.51	59.02	131.42	2.26
CV (%)[3]	9.37	--	--	9.69	--	--	7.66	--	--
P-valor[4]	0.21	--	--	0.64	--	--	0.51	--	--

G1: group inoculated with phage M13. G2: Group inoculated with *E. coli* ER2738 infected by the phage M13; G3: Group infected by *E. coli* ER2738; G4: Group inoculated with PEG; G5: Group inoculated with PBS. The birds of G1 were inoculated at all ages (10 PFUs/bird). The birds of G3 were inoculated at 2 days (5.04 log CFU/animal), 8 days (6.64 log CFU/animal) and 15 days of age (6.69 log CFU/animal). [1]Means corresponding to the experimental treatments; [2]MDFC and FA data are descriptive only; [3]CV (%) corresponds to the coefficient of variation; [4]Statistical probability; no differences were found for the MDWG variable (P > 0.05) by the Tukey test.

Figures

Figure 1.

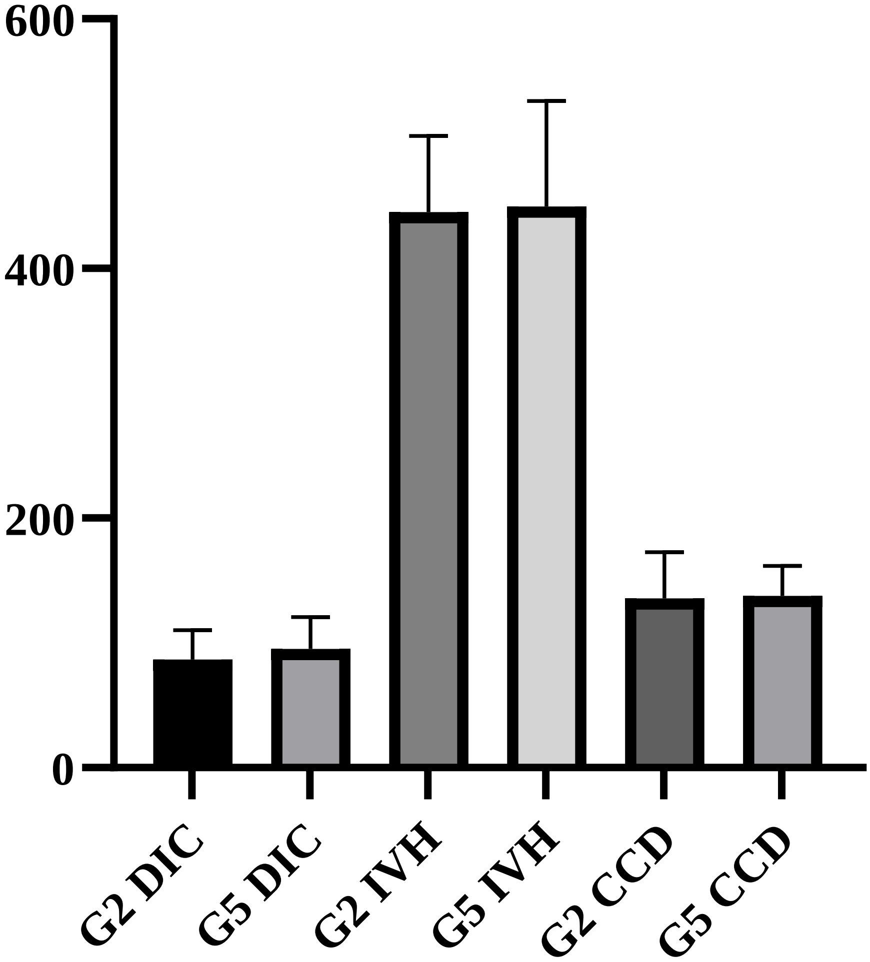
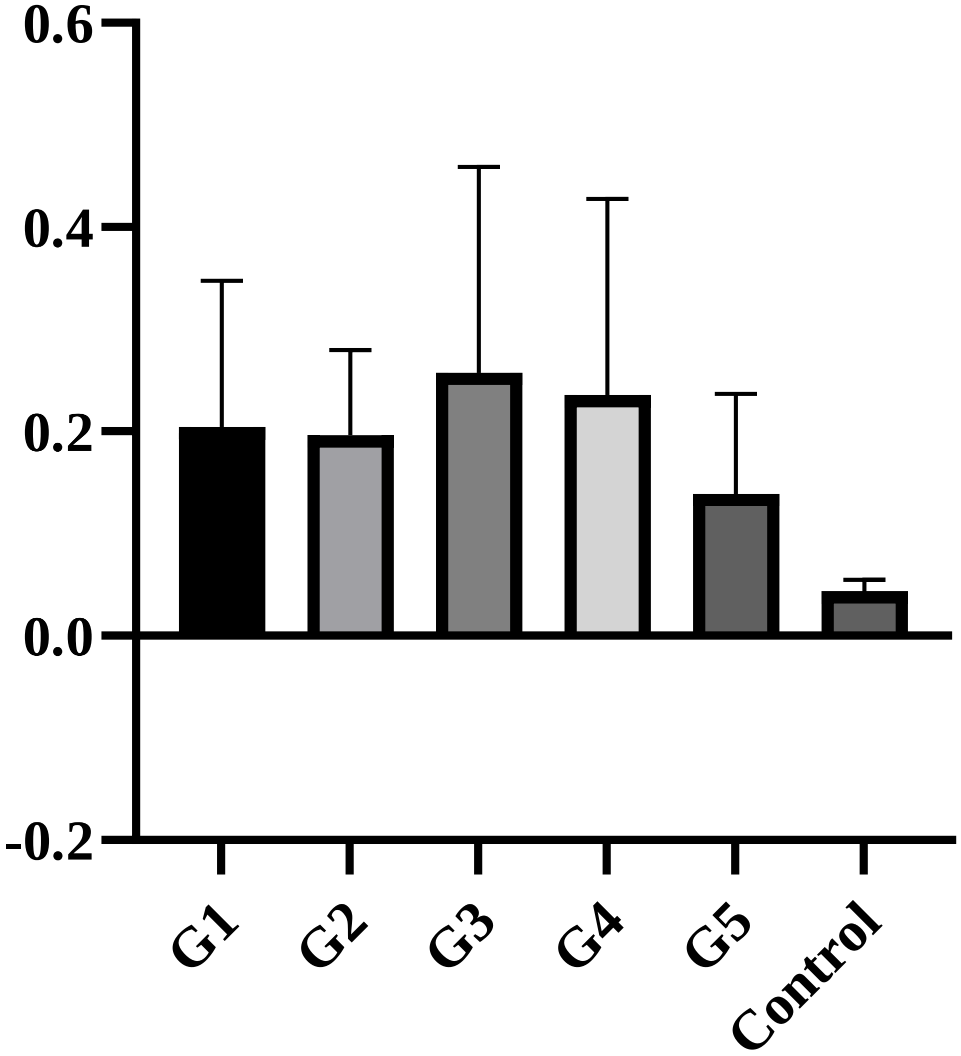


Figure 2.



Supplementary Material

Supplementary Table 1. Nutritional requirements of birds and vitamin and mineral supplementation.

NUTRIENT	Initial 1–7 (days old)		Fattening 7–32 (days old)		Finishing 33–42 (days old)	
Metabolisable energy (Kcal/kg)[1]	3.000		3.100		3.100	
Crude protein (%)[1]	20.79		19.41		19.41	
Calcium (%)[2]	0.88		0.82		0.82	
Phosphorus (%)[2]	0.44		0.41		0.41	
Sodium (%)[2]	0.21		0.21		0.21	
Potassium (%)[2]	0.59		0.59		0.59	
Chlorine (%)[2]	0.19		0.18		0.18	
Linoleic acid (%)[2]	0.63		0.57		1.11	
Amino acid[2]	Dig.	Total	Dig.	Total	Dig.	Total
Lysine (%)[2]	0.98	1.07	0.88	0.96	0.76	0.83
Methionine (%)[2]	0.44	0.47	0.40	0.44	0.36	0.38
Methionine + cystine (%)[2]	0.74	0.83	0.67	0.75	0.59	0.67
Tryptophan (%)[2]	0.18	0.21	0.17	0.20	0.15	0.18
Threonine (%)[2]	0.66	0.77	0.60	0.70	0.52	0.62
Arginine (%)[2]	1.05	1.13	0.94	1.01	0.81	0.87
Valine (%)[2]	0.73	0.80	0.69	0.76	0.61	0.67
Isoleucine (%)[2]	0.71	0.76	0.65	0.70	0.57	0.61
Vitamin A (UI/kg)[2]	12,000		10,000		9,000	
Vitamin D3 (UI/kg)[2]	5,000		4,500		4,000	
Vitamin E (UI/kg)[2]	80		65		55	
Vitamin K (mg/kg)[2]	3.2		3.0		2.2	
Vitamin C (mg/kg)[2]	50		50		25	
Folic acid (mg/kg)[2]	2.2		1.9		1.6	
Biotin (mg/kg)[2]	22		18		15	
Choline (mg/kg)[2]	1,700		1,600		1,550	
Manganese (mg/kg)[2]	120		120		120	
Zinc (mg/kg)[2]	110		110		120	
Iron (mg/kg)[2]	20		20		20	
Copper (mg/kg)[2]	16		16		16	
Selenium (mg/kg)[2]	0.30		0.30		0.30	
Iodine (mg/kg)[2]	1.25		1.25		1.25	

[1] Levels calculated according to the recommended formulation

[2] Levels calculated according to the genetic lineage recommendation (Hi-Line 2016)

Ingredient compositions of initial (1–7 days old), fattening (7–32 days old) and finishing (33–42 days) diets for birds.

INGREDIENTS[1]	Initial (g/kg) 1–7 (days old)	Fattening (g/kg) 7–32 (days old)	Finishing (g/kg) 33–42 (days old)
Corn meal 7.88%	593.2	614.3	614.3
Soybean meal 45%	344.3	308.4	308.6
Soybean oil	23.00	32.6	32.7
Dicalcium phosphate	18.00	16.7	16.7
Limestone	9.00	8.4	8.4
DL-Methionine 99%	1.55	1.55	1.34
L-Lysine HCl 78.4%	1.85	2.03	2.03
L-Threonine 98.5%	0.5	0.5	0.5
Choline chloride 70%	0.5	0.5	0.5
Sodium chloride	4.95	4.7	4.7
Anticoccidian[2]	--	--	--
Antibiotic[2]	--	--	--
Vitamin/mineral supplement	3.0	3.0	3.0
Total (g)	1,000	1,000	1,000

[1] Levels calculated according to the genetic lineage recommendation (Hi-Line 2016)

[2] Not included for influencing the results

Supplementary Table 2. Quantification of ECR and phage M13 inoculated in birds and phage M13 isolated from bird faeces.

Initial inoculum of infected bacterium		**Quantification of phage M13 in faeces**		**Difference between last inoculum and phage M13 in faeces**
Age (days)	**log CFU/bird**	**Age**	**log PFU/g of faeces**	**Log CFU**
2	5.04	7	3.93	1.11
8	6.64	14	4.02	2.62
15	6.69	21	4.36	2.33
		42	3.40	3.29

ECR: *E. coli* ER2738V